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Changing fitness consequences of *hsp70* copy number in transgenic *Drosophila* larvae undergoing natural thermal stress

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Summary

1. Transgenic manipulation of the gene copy number of *hsp70*, which encodes the major inducible heat-shock protein of *Drosophila melanogaster* (Hsp70), affects both Hsp70 levels and inducible thermotolerance in the laboratory; here parallel effects in transgenic *Drosophila* larvae undergoing natural or simulated natural thermal stress are demonstrated.

2. Necrotic fruit was infested with larvae of either of two transgenic strains, one transformed with 12 extra copies of the *hsp70* gene (extra-copy strain) and a sister strain possessing only the wild-type number (10) of *hsp70* genes (excision strain), and then allowed to heat to variable extents.

3. As the intensity of thermal stress increased, the consequences of extra *hsp70* copies reversed. After no or moderate thermal stress, excision larvae survived better than did extra copy larvae. By contrast, extra copy larvae tolerated intense hyperthermia better than did excision larvae.

4. These results establish that the Hsp70-mediated enhancement of stress tolerance, previously demonstrated only for artificial stress regimes in the laboratory, extends to natural stress regimes.

5. Mortality due to overexpression of Hsp70, however, also increases under mild natural stress regimes, buttressing the ecological relevance of a hypothesized evolutionary trade-off of the benefits and adverse consequences of Hsp70 expression.

Key-words: Heat-shock proteins, Hsp70, temperature

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Introduction

Hsp70, the major inducible heat-shock protein (Hsp) of *Drosophila melanogaster* Meigen, is encoded by 10 nearly identical diploid gene copies in this species and belongs to a group of proteins (molecular chaperones) whose functions include coping with stress-damaged proteins (Feder & Krebs 1998; Feder 1999). Laboratory studies of transgenic *Drosophila* have shown that in whole organisms this single protein accounts for a significant fraction of inducible tolerance of high temperatures (Welte *et al.* 1993; Feder *et al.* 1996; Krebs & Feder 1998b); in the absence of stress and at very high concentrations, however, Hsp70 is inimical to development, growth and survival (Krebs & Feder 1997a; 1998b). This relationship may constitute a trade-off that limits the maximum level of Hsp70 expression (Krebs & Feder 1997a,b; 1998a,b; Krebs, Feder & Lee 1998).

Attempts to extrapolate such work with transgenic strains in the laboratory to populations in nature face two principal challenges. First, in nature traits such as Hsp70 expression could be so highly conserved that no variation exists upon which selection can operate (Krebs & Feder 1997b; Bettencourt, Feder & Cavicchi 1999). Studies of a focal natural population have now revealed, however, that isofemale lines covary in Hsp70 expression, inducible thermotolerance and mortality in the absence of stress as do transgenic Hsp70 mutants, although the magnitude of variation is not as large for wild-derived *Drosophila* as in the transgenic strains (Krebs & Feder 1997b; Krebs *et al.* 1998). Similar patterns occur in wild-type strains undergoing laboratory evolution (Bettencourt *et al.* 1999).

The second challenge is that laboratory studies often cannot duplicate natural variation in stress (Feder &

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Krebs 1997; Dahlgaard *et al.* 1998; Feder & Hofmann 1999). In the wild, non-adult *Drosophila* infest necrotic fruit, in which they encounter diverse patterns of thermal stress that are harmful if not lethal (Feder 1997; Feder, Blair & Figueras 1997). For example, larvae infesting necrotic apples and peaches in semi-natural sites in temperate North America may encounter monotonically but gradually increasing temperatures that become lethal 60–90 min after fruits become sunlit, irregular but sub-lethal warming, and irregular peaks and troughs of temperature as weather changes (Feder 1997; Feder *et al.* 1997). Also, in nature larvae undergo this stress exposure while in or on necrotic fruit, which is unlike the containers in which laboratory thermal stress is administered. Because of such differences between the field and laboratory, the ecological and evolutionary relevance is unclear for laboratory results on Hsp70's impact on inducible thermotolerance and potential trade-offs of Hsp70's benefits and disadvantages. For this reason, we have again examined inducible thermotolerance and evidence for a trade-off in an *hsp70* allelic series, but for larvae undergoing natural or simulated natural thermal stress in necrotic apples. This examination shows that for both laboratory and natural thermal stress, transgenic elevation of Hsp70 expression benefits larvae undergoing severe thermal stress but is disadvantageous in larvae undergoing lesser or no thermal stress.

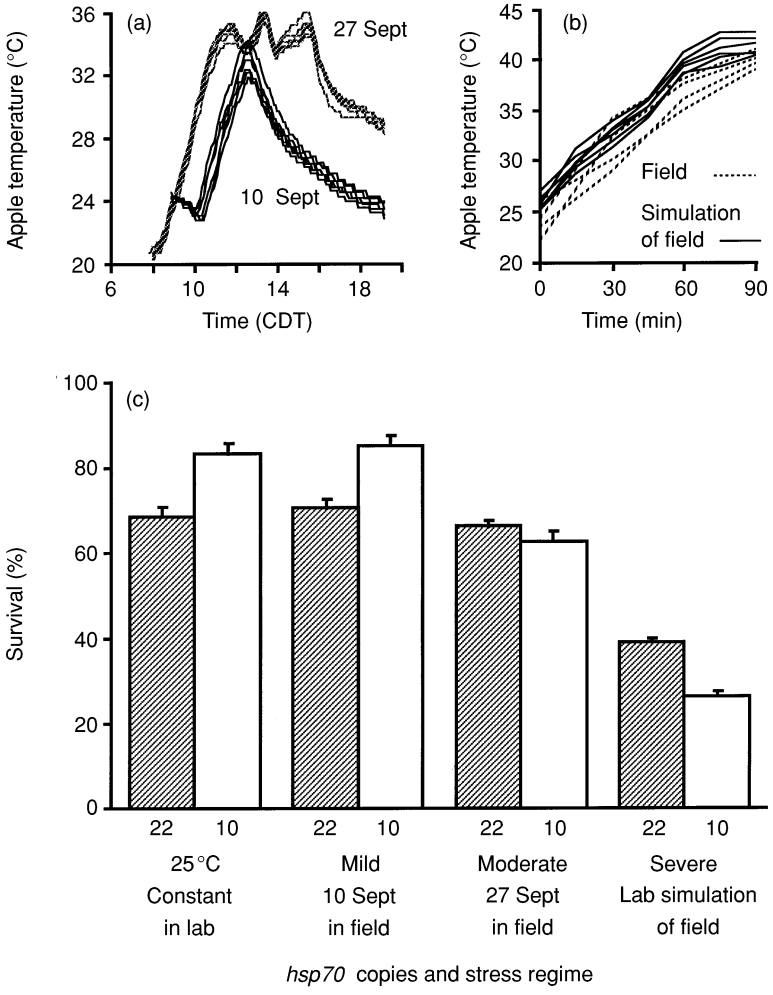

Fig. 1. (a) Temperatures of necrotic apples containing transgenic *D. melanogaster* larvae placed outside on the University of Chicago campus on 10 and 27 September 1998. (b) Temperatures of necrotic apples under simulated insolation (solid line) and of insolated necrotic fruit in an orchard in northern Indiana (broken line), as previously reported (Feder 1997; Feder *et al.* 1997). (c) Larvae-to-adult survivorship of *D. melanogaster* larvae possessing either 10 or 22 *hsp70* gene copies from necrotic apples exposed to constant 25 °C and to these thermal regimes. Means + standard errors are plotted.

Methods and materials

Effects of *HSP70* gene copy number and natural hyperthermia on larvae-to-adult survival

Larva-to-adult survival following exposure to natural thermal conditions was compared in an allelic series of *hsp70* mutants (extra copy = 12 transgenic *hsp70* copies + 10 wild type copies; excision = 10 wild type copies only) created by homologous recombination (Welte *et al.* 1993). In both strains, Hsp70 levels are undetectable in *Drosophila* maintained at 25 °C, while larvae and pupae of the extra copy strain accumulate greater amounts of Hsp70 upon heat shock than the excision strain (Feder *et al.* 1996; Krebs & Feder 1997a, 1998b; Tatar, Khazaeli & Curtsinger 1997). Stocks were reared on yeasted molasses–cornmeal medium at 25 °C. Size-matched red apples (*Malus domestica*) were bruised on one side and allowed to necrotize for 5 days at 25 °C after removal of a 300-mm^2 skin patch from the bruised area. In the laboratory, 50 excision or extra copy larvae in the first day of the third instar were placed on each apple where the skin had been removed. Each apple was then placed in a 1-l cylindrical container of fine mesh, which excluded other insects from the apples. Approximately 12 h later, between 0800 and 0900 CDT, these apples (n = 6–8 for each genotype) were placed out of doors on a grass lawn on the campus of the University of Chicago, a botanical garden planted with grass and a variety of species of herbaceous and woody plants. This experiment was repeated on two days, 10 and 27 September 1998. Figure 1(a) presents the corresponding thermal regimes within the necrotic apples. Local weather conditions on these dates are available from the National Oceanic & Atmospheric Administration (1998). In the field, apple temperatures were monitored every 5 min with Stowaway XTI thermocouple dataloggers (Onset Computer Corporation, Pocasset, MA, USA). The external sensor, a $2{\cdot}5 \times 20$ mm^2 cylinder connected to the datalogger via 2-mm diameter cable, was placed just underneath the skin of the necrotic apple near where the skin had been removed. Sensor mass was < 1% of apple mass. After ≈ 8 h of exposure to field temperatures, infested apples were returned to the laboratory and placed at 25 °C for subsequent development of surviving larvae. Eclosing adults were censused daily. A preliminary experiment confirmed that the mesh containers do not affect the temperatures of apples within.

EFFECTS OF *HSP70* GENE COPY NUMBER AND SIMULATED EXTREME NATURAL HYPERTHERMIA ON LARVAE-TO-ADULT SURVIVAL

In nature, *Drosophila* larvae inhabiting necrotic fruit in direct sunlight can experience heating rates as high as 0·17 °C min^{-1} and temperatures exceeding 40 °C on warm summer days with few clouds (Feder 1997; Feder *et al*. 1997). Because culture of experimental larvae and necrotic fruit could not be synchronized with such weather (i.e. autumn and winter arrived), a laboratory protocol was developed that replicated the thermal kinetics of necrotic fruit exposed to continuous sunlight on a warm summer day. When necrotic apples were placed in a Blue M Laboratory Drying Oven (Blue M, Blue Island, IL, USA) set at 42 °C, their heating closely resembled (Fig. 1b) that previously described for necrotic apples on warm summer days (Feder 1997; Feder *et al*. 1997). Thirty larvae in the second instar from both the excision and extra copy strains were placed into each of 12 size-matched necrotic apples. Six of these apples were placed in an oven as described above for 90 min and returned to a 25 °C incubator for the remainder of the larval development. The other six apples were placed in the drying oven set at 25 °C for 90 min and afterwards returned to the 25 °C incubator. While in the oven, fruit temperatures were monitored with Stowaway XTI thermocouple dataloggers as described above. Eclosing adults were identified as either extra copy or excision strain based on eye colour (red in extra copy, white in excision) and censused daily.

Results

Experimental thermal regimes ranged from relatively benign (control apples maintained in the laboratory at 25 °C; hereafter 'control') to progressively more rapid heating and more exposure to high temperatures (Fig. 1). On 10 September (hereafter 'mild'), apples reached maximum temperatures ranging from 32 to 36 °C but thereafter cooled owing to the arrival of a cold front. On 27 September (hereafter 'moderate'), which was partly cloudy, apple temperatures varied between 33 and 36 °C for ≈ 5 h, after which they cooled due to shading by a large tree. Summer ended before the experiment could be repeated on a very warm day, and so an intense natural thermal regime was simulated with a laboratory oven. Apples exposed to oven-heating (hereafter 'intense') had warm-up rates (0·17 °C min^{-1}) and maximum temperatures (40–43 °C) nearly identical to values previously reported for insolated necrotic fruit (Fig. 1b; Feder 1997; Feder *et al*. 1997). The average mortality of all larvae (i.e. regardless of strain) was similar after the 'control' and 'mild' regimes, increased slightly in the 'moderate' regime, and was greatest in the simulated 'intense' stress regime (Fig. 1c).

The number of diploid copies of the *hsp70* gene significantly affected the relative survival of *Drosophila* larvae, but the magnitude and direction of strain differences were dependent on the thermal regime (Table 1, Fig. 1c). In larvae undergoing either control or mild stress, excision larvae with 10 *hsp70* copies survived significantly better than extra-copy larvae with 22 *hsp70* copies (Fig. 1c; Tukey–Kramer HSD, $P < 0·05$). In larvae undergoing moderate stress, both strains had similar mortality (Fig. 1c). This change in survival after mild stress was due to the marked increase in mortality of excision larvae; mortality of extra copy larvae was similar after control, mild and moderate thermal stress. Finally, after intense simulated stress, extra-copy larvae survived significantly better than excision larvae.

The likely demographic consequences of *hsp70* copy number under changing intensities of natural thermal stress are evident from the corresponding relative survival rates of the two genotypes. After control and mild stress, the relative survival of excision larvae is greater than that of extra copy larvae. With increasing levels of thermal stress, this relationship reverses, until extra copy larvae have a greater relative survival than excision larvae after intense simulated stress.

Table 1. Analysis of variance for effect of stress intensity (control, mild, moderate, severe simulated in laboratory) and *hsp70* copy number on larval survival

Variable	df	Sum of squares	Mean square	*F*	*P*
Stress intensity	3	16912·9	5637·6	105·0	< 0·0001
hsp70 copy number	1	29·325	29·3	0·5	0·4641
Stress X copy number	3	1324·9	441·6	8·2	0·0002
Residual	40	2146·7	53·7		

Discussion

The distinction between laboratory and natural thermal regimes has long posed a challenge to functional ecologists and ecophysiologists. In terms of temperature, laboratory studies permit exquisite experimental control but usually impose thermal regimes that lack ecological relevance and exclude many behaviours with thermal consequences, whereas studies of unrestrained organisms in nature often cannot rigorously establish the significance of physiological and biochemical mechanisms relevant to thermal stress (Burggren 1987). Accelerating advances in telemetry and computer-controlled experimentation have been one response to this challenge. As demonstrated in the present study, another response is to choose a study system that is physically transportable between laboratory and field environments. The necrotic fruit–*Drosophila* system can be fully manipulated in either laboratory or field, as can the phenotypic traits of the study organism and all aspects of its abiotic and biotic environment in nature (Feder 1997). In this system, the distinction between laboratory and field can become trivial.

Because of the tractability of this system, we have been able to take a step that has seldom been possible in animal thermal ecophysiology: the direct testing of an experimentally manipulated thermal phenotype in the natural setting. Although our results are uniformly confirmatory of laboratory findings (i.e. upon laboratory, natural or simulated natural thermal stress, the net advantage of transgenic augmentation of *hsp70* copy number varies directly with the intensity of stress), our ability to repeat them in the natural setting provides confidence in their ecological and evolutionary relevance that would otherwise be lacking.

Two additional findings are necessary to establish the ecological and evolutionary relevance of these findings. First, Hsp70 protein expression needs to be variable and heritable within natural populations, in which the genetic manipulations used to create the transgenic strains have no counterpart. Prior work with a focal natural population, however, demonstrates that a natural population can meet both of these requirements (Feder *et al*. 1997; Krebs *et al*. 1998), and laboratory populations undergoing experimental evolution have evolved differences in thermotolerance, Hsp70 expression, and in the sequence of several of the genes that encode Hsp70 (Bettencourt *et al*. 1999; B. R. Bettencourt, unpublished data). The *hsp70* genes themselves, moreover, have undergone duplication during the evolution of the *Drosophila melanogaster* species group (Leigh-Brown & Ish-Horowicz 1981), although *Drosophila melanogaster* itself is not known to be polymorphic for *hsp70* copy number (Feder & Hofmann 1999). Second, thermal stress needs to be frequent in occurrence and variable in intensity in nature. Prior studies of necrotic fruit and indwelling *Drosophila* establish that natural thermal stress does occur in this context and can vary considerably in intensity (Feder 1997; Feder *et al*. 1997; Dahlgaard *et al*. 1998), but not the exact probability of encountering any given level of thermal stress in a natural *Drosophila* population. This probability can be estimated from meteorological records and biophysical models of *Drosophila* oviposition sites (Feder *et al*. 1999), and an attempt at such an estimate is now in progress.

Thus, these findings buttress an emerging conclusion as to the limits of Hsp70 expression in organisms. Except in severe thermal stress, where Hsp70's contribution to stress tolerance is most important, Hsp70 is apparently a liability for organisms that express it because it is toxic, expensive to produce, or both (see Krebs & Loeschcke 1994; Heckathorn *et al*. 1996a,b; Xia *et al*. 1999; and references cited in the Introduction). These benefits and liabilities may trade off in evolving a lower level of Hsp70 expression than that specified by Hsp70's benefits alone. Although laboratory studies furnished the original evidence for such a trade-off, our findings for natural stress regimes support this scenario in every respect. We predict therefore that populations undergoing regular exposure to sublethal Hsp70-inducing stress should evolve lower than average levels of Hsp70 expression, and only populations undergoing regular exposure to severe stress should evolve higher than average levels of Hsp70 expression (Krebs *et al*. 1998).

Finally, we have restricted our examination to a single episode of natural or simulated natural stress, temperature, and a single component of fitness, survival from larva to adult eclosion. While natural thermal stress could occur only once during larval development, as in our imposed stress regimes, it could also occur multiple times (Feder *et al*. 1997). Natural stress could well be multifactorial, comprising competition, predation, parasitism/infection, desiccation of necrotic fruit, etc. The *Drosophila* larva–necrotic fruit system is also amenable to systematic manipulation of each or all such variables. Additional components of fitness are also manipulatable and observable in such systems. For example, the same natural thermal regimes that reduced survival in the present study also increase the incidence of morphologically abnormal adults (Roberts & Feder 1999), which can mate poorly if at all (J. Posluzny and M. Feder, unpublished data). Alternatively, non-lethal thermal stress of larvae can actually enhance the fitness of adult *Drosophila simulans* infected with the endosymbiont *Wolbachia* (Feder *et al*. 1999).

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